JOURNAL OF VIROLOGY, Sept. 1997, p. 6641–6649
0022-538X/97/$04.00+0

Vol. 71, No. 9

Highly Efficient and Sustained Gene Transfer in Adult Neurons with a Lentivirus Vector

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Received 12 March 1997/Accepted 20 May 1997

The identification of monogenic and complex genes responsible for neurological disorders requires new approaches for delivering therapeutic protein genes to significant numbers of cells in the central nervous system. A lentivirus-based vector capable of infecting dividing and quiescent cells was investigated in vivo by injecting highly concentrated viral vector stock into the striatum and hippocampus of adult rats. Control brains were injected with a Moloney murine leukemia virus, adenovirus, or adeno-associated virus vector. The volumes of the areas containing transduced cells and the transduced-cell densities were stereologically determined to provide a basis for comparison among different viral vectors and variants of the viral vector stocks. The efficiency of infection by the lentivirus vector was improved by deoxynucleoside triphosphate pretreatment of the vector and was reduced following mutation of integrase and the Vpr-matrix protein complex involved in the nuclear translocation of the preintegration complex. The lentivirus vector system was able to efficiently and stably infect quiescent cells in the primary injection site with transgene expression for over 6 months. Triple labeling showed that 88.7% of striatal cells transduced by the lentivirus vector were terminally differentiated neurons.

The application of molecular genetics to human biology and disease has improved our understanding of and ability to treat a variety of diseases. Somatic gene therapy with the introduction of recombinant genes requires efficient gene transfer, either via viral or nonviral methods. Despite the progress in vitro and in vivo in this field during the last decade, clinical trials involving patients with a variety of disorders have not led to successful treatment protocols (21, 22, 33, 63). Problems with effective gene transfer include loss of transgene and immune responses directed against transgene and viral protein expression by adenovirus vectors (10, 16, 17), the limitation of retroviral gene transfer in dividing cells, and the low in vivo efficiency of gene transfer with adeno-associated virus vectors (1, 28, 48).

Any successful form of gene therapy must combine not only an appropriate disease target but also a gene delivery system that is highly efficient on a large scale and guarantees long-term expression without toxicity. Retroviral vectors based on the Moloney murine leukemia virus (MLV) have been the most commonly used vectors for gene transfer into the host cell genome because they can be rendered replication incompetent, they stably integrate into the host cell genome, and they do not express any viral proteins (6, 47). However, gene transfer to the central nervous system, targeting a large majority of neurological diseases, requires the transduction of terminally differentiated neurons. Postmitotic neurons are generally refractory to stable infection by retroviral vectors, which require the breakdown of the nuclear membrane during cell division in order to insert the transgene into the host cell genome (44). Lentivirus vectors based on the human immunodeficiency virus (HIV) represent a more complex form of retrovirus that can infect and stably transduce dividing as well as terminally differentiated cells (49, 50) (Fig. 1). High-titer lentivirus vector stocks are obtained through transient transfection protocols, as described for retrovirus vectors (51, 53). The stability of the viral vector preparation during concentration and the range of perceptible target cells are increased by pseudotyping the lentivirus vector with the envelope G protein of the vesicular stomatitis virus (VSV-G) (8).

We have injected highly concentrated lentivirus vector stocks into the striatum and hippocampus of adult rats and determined the level of transgene expression for up to 6 months as well as the transduction efficiency. We used modified stereological methods to allow comparison among the HIV adeno-associated virus (AAV), adenovirus (ADV), and MLV vectors with respect to the duration and pattern of transgene expression. Modification of the lentivirus vector preparation by adding four deoxynucleoside triphosphates (dNTPs) in vitro results in increased infectivity in vivo, whereas mutations in integrase or nuclear localization signal significantly decrease the efficiency.

MATERIALS AND METHODS

Plasmids and viral vector constructions. The lentivirus vector was produced by cotransfection of human kidney 293T cells with three plasmids by using the methods of Pear et al. and Naviaux et al. (51, 53). Construction of the lentivirus-derived plasmid (pHR'CMV) driven by the human cytomegalovirus (hCMV) promoter carrying the transgene for β-galactosidase (β-Gal) or green fluorescent protein has been described (49, 50). In the pHR'CMV plasmid large portions of the envelope coding sequence were deleted. The integrase mutant variant of the lentivirus vector was generated by introduction of a single defect into the catalytic domain (aspartato-to-valine change at position 64) of integrase (36, 49). The packaging plasmid (pCMVΔR8.2) provided all vector proteins driven by the hCMV promoter but the envelope protein; the envelope protein was encoded by the third plasmid (pMD.G), which provided the heterologous vesicular stomatitis virus envelope. The packaging plasmid mutation in the Vpr-matrix nuclear localization signal complex (Vpr/MA NLS) was achieved by transferring the corresponding region of the $MA_{ΔNLS}$ΔVpr proviral DNA into the parental plasmid CMVΔR8.2 (24, 49). The hCMV promoter drives the VSV-G reading frame. Viral vector generation was obtained by cotransfection of 293T cells on 10-cm plates with 15 μg of pCMVΔR8.2, 20 μg of either pHR'CMVLacZ or pHR'CMVGFP (green fluorescent protein), and 5 μg of pMD.G by calcium

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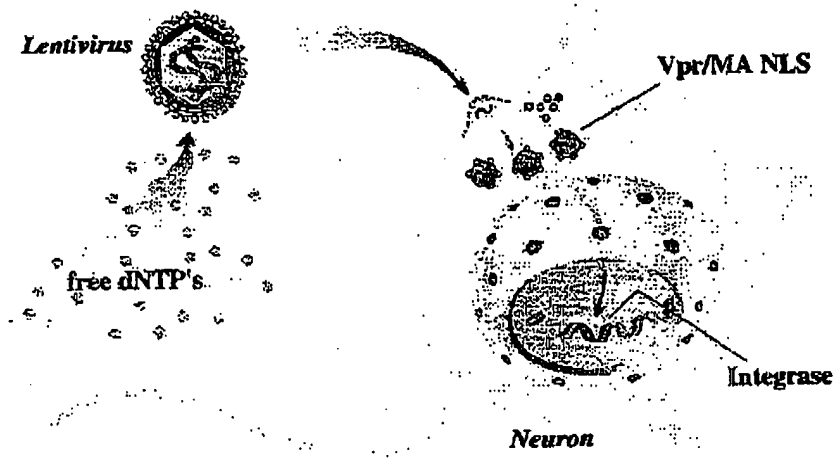


FIG. 1. Lentivirus nuclear import mechanism. The recognition of the uncoated lentivirus nucleoprotein complex by the cell nuclear import machinery allows the active transport of viral genome into the nucleus through the nucleopore and stable integration into the target cell genome. This mechanism enables lentiviruses to infect nondividing cells. Reverse transcription is promoted and there is a subsequent increase in infection efficiency following the addition of the four dNTPs in vitro.

phosphate precipitation (13). After 62 h the conditioned medium was harvested, low-speed centrifuged, and filtered through a 0.45-μm-pore-size filter. p24 antigen was detected with an enzyme-linked immunosorbent assay kit (Dupont). To obtain titers of the viral vector stocks, fibroblasts (208F) were transduced with serially diluted supernatants. After medium change and further incubation for 36 h, the expression of β-Gal was scored by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining. Further vector concentration was achieved by ultracentrifugation at 50,000 × *g* for 90 min, resuspension in Tris-buffered saline (TBS) containing 10 mM $MgCl_2$ pooling, and incubation with and without the four dNTPs (0.1 mM each), 3 mM spermine, and 0.3 mM spermidine for 2 h at 37°C. After the second ultracentrifugation the pellet was resuspended in sterile saline with 2 μg of Polybrene per ml. The MLV-based β-Gal-expressing vector was similarly generated in 293T cells and pseudotyped with the VSV-G envelope. The adenovirus vector was a second-generation vector (17), and β-Gal-expressing adeno-associated virus vector was a generous gift from Somatix (Alameda, Calif.). The transgene expressing β-Gal was driven by the hCMV promoter. Every viral vector (HIV, HIV mutant, AAV, ADV, MLV) was tested on 208F fibroblasts in six serial dilutions of viral vector stock (1 to 10^{-6} μl of vector per well) and the viral titer was determined by counting the number of foci of X-Gal-containing blue cells per well divided by the dilution factor.

For safety purposes every single batch of lentivirus vector was tested for the absence of replication-competent viral vectors (12).

In vivo experiments. Adult female Fischer 344 rats were anesthetized intramuscularly (44 mg of ketamine per kg of body weight, 0.75 mg of acepromazine per kg, and 4 mg of xylazine per kg in 0.9% NaCl), and 2 μl of the viral-vector concentrated stock (2×10^8 to 4×10^8 transducing units [TU]/ml) was injected into the striatum (anterior posterior [AP], +0.2; medial lateral [ML], ±3.5; dorsal ventral [DV], −4.5) and the hippocampus (AP, −3.5; ML, ±3.0; DV, −4.0) bilaterally with a 5-μl Hamilton syringe. After 2, 6, 12, and 24 weeks the animals were sacrificed and perfused intracardially with saline, 4% paraformaldehyde, and 0.2% glutaraldehyde. The brains were removed, postfixed, and saturated in 30% sucrose. The brains were then frozen and sectioned on a sliding microtome into 50-μm slices. Primary antibodies raised in three different species were pooled in TBS with 10% donkey serum and 0.3% Triton X-100 and incubated for 48 h at 4°C. The antibody for β-Gal (rabbit polyclonal antibody [Cortex, Irvine, Calif.]; 1:5,000) was combined with two of the following antibodies: NeuN (mouse monoclonal antibody [generous gift of R. J. Mullin]; 1:20), GFAP (guinea pig polyclonal antibody [Advanced Immunochemicals]; 1:250), ED1, OX8, CD4 (mouse monoclonal antibody [Chemicon]; 1:1,000), tyrosine hydroxylase (mouse monoclonal antibody [Chemicon]; 1:2,000), ChAT (goat polyclonal antibody [Chemicon]; 1:200), and RIP (mouse monoclonal antibody in Dulbecco modified Eagle medium supernatant [generous gift of S. Hockfield]; 1:20). Sections were washed and blocked in TBS with donkey serum. Corresponding secondary antibodies (donkey anti-mouse biotin, donkey anti-rabbit fluorescein isothiocyanate, donkey anti-guinea pig Cy5, and donkey anti-goat Cy5 [Jackson, ImmunoResearch, West Grove, Pa.]; 1:250) were pooled, and sections were incubated for 2 h at room temperature, followed by washing in TBS and 2 h of incubation at room temperature in streptavidin Cy3 or streptavidin Texas red in TBS (1:250; Jackson). The sections were analyzed by confocal scanning laser microscopy (Bio-Rad). The signals were collected, digitally color enhanced, and superimposed.

Every sixth section through the brain was stained immunohistochemically for β-Gal. The number of positive cells in the striatum was quantified by a modified stereological procedure. The profile of the striatum for each section was determined in a systematic, uniform random manner applying the optical disector procedure (54, 60), which provides a numeric cell density (cells per area). The volume of the striatal injection site, with the midpoint of the sample centered at the injection site, was calculated by the Calveri procedure (60). To determine the nature of transduced cells, triple-labeled sections were scanned with the confocal laser microscope, and a representative sample of 100 transduced cells was examined for colocalization of β-Gal with either NeuN or GFAP. The bilateral injection sites were pooled, as were those of the corresponding groups (2, 6, 12, and 24 weeks), and the result was expressed as the percentage of β-Gal-transduced cells that were NeuN positive. Data were analyzed with Stat.View 4.01 for Macintosh and multiway analysis of variance (ANOVA), followed by a Fisher post hoc test ($P < 0.05$).

RESULTS

Sustained transgene expression. Two microliters of titer-matched vector stocks (1×10^6 to 3×10^8 TU/ml) were injected into the striatum and hippocampus, and transduction was evaluated 2, 6, 12, and 24 weeks later. Light-microscopic evaluation of the 32 striatal injection sites (SIS) showed transduced, β-Gal-positive cells in every lentivirus vector SIS. The cell spread around the injection site for the lentivirus vector group was 3 to 4 mm, with 30 to 42 positive sections throughout the striatum (Fig. 2, top panel). To obtain comparable data we used modified stereological methods to evaluate the transduced-cell area volume, providing data about vector spread and cell density and determining the number of cells in the reference areas. The 32 hippocampal injection sites were excluded from the quantitation because of the wide and variable spread of transduced cells along Ammon's horn (Fig. 2, bottom panel). The average volume of the transduced-cell area around the SIS for the lentivirus vector was 9.0 mm^3 (standard error of the mean [SEM], ±1.5 mm^3) (Fig. 3A), with no decrease over the 6 months. The cell density by area count was 2,736 cells (SEM, ±280 cells), equally stable over time (Fig. 3B). Transduction-cell area volumes and cell densities for eight striata injected with titer-matched AAV vectors (3.3 mm^3 [SEM, ±0.7 mm^3]; 241 cells [SEM, ±42 cells]), 16 striata injected with

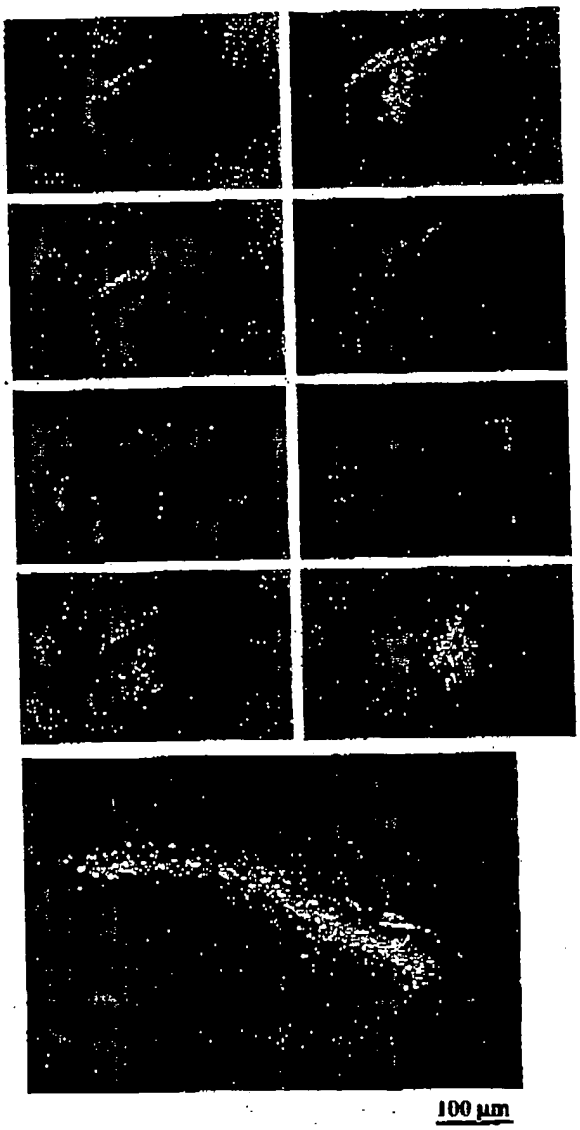


FIG. 2. (Top) Series of one of six coronal sections (50 μm) of a single rat brain unilaterally injected with 2 μl of lentivirus vector expressing green fluorescent protein (GFP) 6 weeks after injection. There is spread of transduced striatal cells over seven sections in the medial striatum, subventricular zone, and corpus callosum. Low-magnification images of horizontal sections (50 μm), triply labeled for β-Gal (green), NeuN (red), and GFAP (blue), of typical HIV vector and β-Gal injection sites 6 months after injection are shown. (Bottom) Confocal microscopy of horizontal sections (50 μm) of hippocampal injection sites of the lentivirus vector 12 weeks after injection. Triple labeling for β-Gal (green); NeuN (red) and GFAP (blue) was used. There is significant spread of transduced cells along Ammon's horn far from the original injection site.

ADV vectors (3.9 mm^3 [SEM, ±1.7 mm^3]; 938 cells [SEM, ±42 cells]), and 16 striata injected with MLV vectors (0.3 mm^3; 55 cells) were significantly lower than in the lentivirus injections, with the exception of those obtained by the ADV vector at the 6-week time point. However, the small number (eight) of injection sites evaluated for the AAV vector precluded further statistical analysis. The low cell density for the MLV group also disallowed further statistical analysis. No cells expressing the transgene could be detected 6 weeks postinjection with the MLV vector. Brains injected with the adenovirus vector (16 SIS) showed a peak of transduced cells and volume 6 weeks postinjection, with a rapid decrease of cells expressing the transgene at late time points. Among the control vectors, only the AAV vector was able to stably transduce cells for up to 6 months; however, the transduction-cell area volume and the density of β-Gal-expressing cells were low (Fig. 3A and B).

Characterization of transduced target cells. The relative proportion of transduced-cell types in the striatum was determined by immunofluorescence staining for a neuronal specific marker (NeuN) that labels terminally differentiated neurons (46), glial fibrillary acidic protein (GFAP) as a marker for astrocytes, as well as additional markers for either tyrosine hydroxylase (TH) or choline-acetyltransferase (Chat). The RIP antibody was used to label oligodendrocytes and Schwann cells (20). The triple labeling and confocal evaluation showed 88.7% (SEM, ±2.5%) of cells labeled with NeuN by the lentivirus vector compared to 66.7% (SEM, ±3.0%) for the AAV vector and 48.5% (SEM, ±6.6%) for the ADV vector. This ratio remained constant over 6 months (Fig. 3C). In the brains injected with the MLV vector no double labeling with NeuN could be detected, confirming the inability of the MLV-based vector to transduce terminally differentiated cells (Fig. 4). Additional immunofluorescence staining of these brains showed smaller, highly branched, transduced cells that were frequently double labeled for RIP or GFAP, which is characteristic of oligodendrocytes and astrocytes transduced by the MLV vector (data not shown).

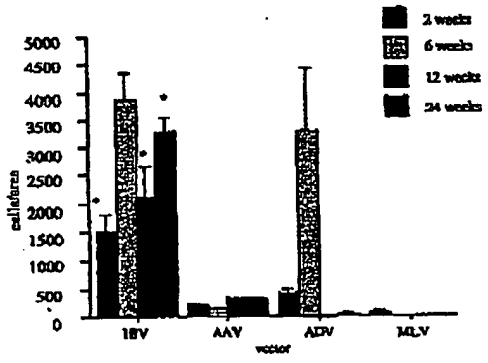
Immune response to viral vector injection in vivo. We examined injection sites of all groups at every time point for invading lymphocytes, macrophages, and astrocytic proliferation to evaluate the immune response in vivo. Two weeks after injection, invading lymphocytes and macrophages could be found in injection sites of all viral vector groups, an extent of infiltration identical to that seen in saline control injection sites. We interpret this to represent the reaction to the mechanical trauma caused by the injections. No further infiltrate was detectable at 6, 12, and 24 weeks in the MLV, AAV, and HIV groups, and transduced cells appeared normal in size and healthy. However, animals injected with the adenovirus vector displayed a significant invasion of OX8-positive T-cytotoxic precursor lymphocytes, fewer CD4 helper-T-cell precursors, and ED1-positive macrophages at all time points. This inflammation was accompanied by significant tissue trauma as evidenced by changes in morphology and cell loss in the area of the injection (Fig. 5A to C). ED1-expressing macrophages and microglial cells were scattered throughout the striatum and hippocampus, and T-cell infiltration of both CD4- and OX8-expressing lymphocytes was found, especially at the 6-week time point, which corresponds with peak transgene expression in the ADV group. We could not detect similar persistent immune responses to the vector or transgene product (β-Gal or green fluorescent protein) in the HIV, AAV, and MLV groups.

Delineation of parameters of vector transduction. Low concentration of the four dNTPs in nondividing cells like neurons is a limiting step in retroviral infection. To investigate the in vivo effect of pretreatment of the viral vector stock with the four dNTPs, SIS were examined 2 and 6 weeks after injection. Because our previous results show consistent transgene expression over time (Fig. 3A and B) with no statistically significant difference between the 2- and 6-week time points, we pooled

A

weeks	HIV	AAV	ADV	MLV
2	3.5	3.1	3.0	0.3
6	11.4	2.3	11.0	0
12	7.8	5.4	0.29	0
24	15.9	2.2	0.9	0

B



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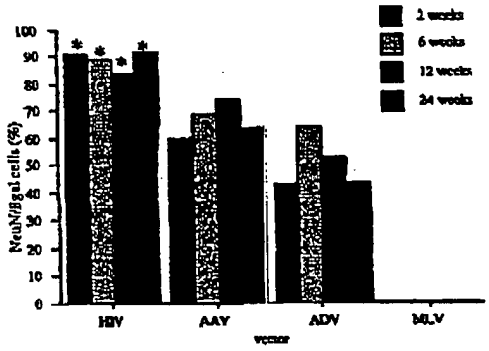

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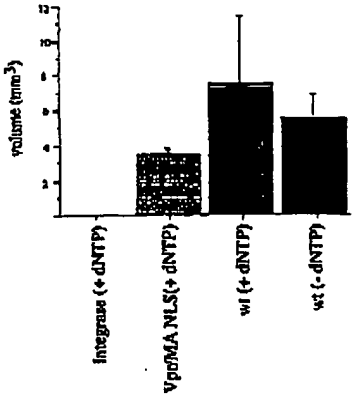

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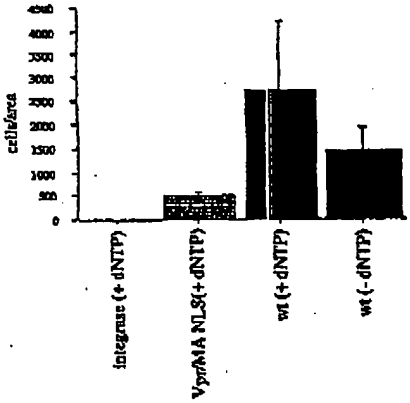

the data for both time points (six SIS of three animals with two SIS each). Each viral preparation was diluted to match the amount of p24 antigen injected in 2 μl of virus preparation. Animals injected with dNTP-pretreated lentivirus vector showed a larger volume (9.0 mm^3; SEM, ±1.4 mm^3) and higher cell density (2,706 cells; SEM, ±270 cells) than animals injected with virus vectors without dNTP pretreatment (4.0 mm^3 [SEM, ±0.6 mm^3]; 1,478 cells [SEM, ±214 cells]), although the difference was not statistically significant (Fig. 3D and E).

The Vpr/MA NLS mutant lentivirus vector, in which both Vpr and the MA NLS were inactivated, still allowed the transfer of the transgene into the target cell but with less efficiency, based on the volume of 2.0 mm^3 (SEM, ±0.3 mm^3) and the cell density of 509 (SEM, ±42 cells) transduced cells in the striatum (Fig. 3D and E). The mutation in the catalytic domain of integrase resulted in a dramatic decrease in cell density (0.5 cells; SEM, ±0.37 cells) and in the volume (0.006 mm^3, SEM ±0.004 mm^3) of the transduced-cell area. For the integrase mutant vector, only single cells around the injection site could be detected, and therefore a statistical evaluation was not possible due to the low total number of transduced cells per injection site. We conclude that integration of lentivirus vector is essential for expression of the transduced gene.

Retrograde transport. Many cells of differing neuronal subtypes expressed β-Gal at each of the primary injection sites. However, β-Gal activity was also detectable in distant secondary brain areas. Fibers labeled for β-Gal were found in the nigrostriatal pathway, and β-Gal-positive cells double labeled for NeuN characterized neuronal cells in the substantia nigra (SN) and ventral tegmental area ipsilaterally. Further staining with antibodies revealed cells expressing the β-Gal transgene and tyrosine hydroxylase in the SN compacta, which is characterized by dopaminergic cells, as shown by confocal microscopy (Fig. 6), suggesting that a few vectors were transported to areas distant from the injection sites.

DISCUSSION

Our data support the in vitro findings that the lentivirus vector is able to transduce efficiently and stably in vivo without a detectable decrease of transgene expression over time. This finding is in contrast to the findings obtained with retroviral vectors based on the Moloney murine leukemia virus, which were able to neither transduce terminally differentiated cells nor express transgenes on a long-term basis in vivo (52, 59, 61), as shown by previous studies with grafts of ex vivo-transduced and -selected cells. Although cells exhibited long-term survival, a loss of transgene expression was observed (15, 52). Perhaps the lentiviruses allow insertion of the viral gene into chromatin domains that are permissive for long-term transgene expression (69).

The low cell density and transduction volume associated with the AAV vector, although contrary to the findings of Kaplitt and others (31, 37), confirm recent reports that reveal a significantly lower transduction efficiency of AAV vectors in primary cell cultures compared to that for retroviral infection (5, 45). The vast majority of AAV vector DNA does not integrate, and the vector-derived message parallels the low transduction efficiency (28, 34). Helper viral functions provided by adenovirus play an important role in the AAV life cycle, specifically in second-strand synthesis (19, 27, 57, 58).

The limitation in long-term transduction by adenovirus vectors that we observed was in accordance with the findings presented in a broad number of publications (2, 3, 30, 62). The immune response as well as the inability of adenovirus vectors to stably integrate the transgene into the host cell DNA results in transgene expression for only 6 weeks.

The difference in the percentage of neurons transduced by the lentivirus vector and by the other viral vectors examined may be the result of differences in the envelope protein as well as an effect of the promoter driving the transgene (the hCMV promoter). The host cell specificity of retroviral vectors can be broadened by replacing the ecotropic or amphotropic envelope protein gene with the VSV-G envelope protein gene of rhabdoviruses (8, 43, 65, 70). These pseudotyped viral vectors do not exhibit a preference for infection of a particular cell type in vitro (42, 55), possibly due to a wide expression of cell surface receptors recognized by the envelope G protein.

The significant prevalence of medium-sized striatal neurons transduced by the lentiviral vector in our study, as opposed to astrocytes, oligodendrocytes, and microglia cells also present in the striatum, may be due to an additional effect of the VSV-G envelope protein and/or preferential expression from the hCMV promoter in neurons, as already shown by Baskar et al. for transgenic animals (4). The MLV viral vector was also pseudotyped with the VSV-G envelope protein and utilized the hCMV promoter to drive transgene expression, but due to its inability to transduce nondividing cells, expression was limited to glia.

Studies on adenovirus gene therapy showed that the immune system plays an important role in determining the duration of expression of the transgene from adenovirus vectors (9, 11, 32, 68). T-cell infiltration of cytotoxic T cells (OX8) as well as activated CD4 (CD4) cells in adenovirus vector-injected brains and livers reflected the immune response to adenovirus proteins expressed by the host cell (9–11, 17). Our results confirm that retroviral vectors (MLV, HIV) as well as the AAV vector (66) do not generate a significant immune response and subsequent loss of transgene expression in vivo. Long-term cellular immune response to β-Gal, the transgene product (as shown by replication-defective herpes simplex virus [7]), or green fluorescent protein in the HIV, AAV, and MLV groups was not observed.

Reverse transcription of retroviral genomic RNA in the target cell is one of the limiting cellular factors in retroviral infection. Recently it has been demonstrated that reverse transcription can be improved by increasing the extracellular con-

FIG. 3. (A) Volume of the area containing transduced cells (mm^3). The results obtained by ANOVA of the transduced-cell area volume in eight SIS for the HIV vector (four animals), two SIS for the AAV vector (one animal), four SIS for the ADV vector (two animals), and four SIS for the MLV vector (two animals) at each time point (2, 6, 12, and 24 weeks) are shown. The AAV vector had to be excluded from statistical evaluation because of the small number of injections (ANOVA and Fisher's procedure for least squares difference; $P < 0.05$). (B) Density of transduced cells. The cell density of the lentivirus vector is significantly different from the control MLV ($P = 0.0093$), AAV ($P = 0.0014$), and ADV ($P = 0.0022$) vectors at all time points evaluated (ANOVA and Fisher's PLSD). (C) Analysis of transduced cells expressing β-Gal that also express NeuN, a marker for terminally differentiated neurons. Results are given as the percentage of the total number of β-Gal-positive cells that detected by confocal microscopy. There is a significant difference between the lentivirus and the ADV ($P = 0.0006$) as well as the AAV vectors ($P < 0.0001$; ANOVA and Fisher's PLSD). Volume of the area containing transduced cells (D) and density of transduced cells (E). Interaction bars for transduced-cell area volume and cell density for the integrase, Vpr/MA NLS mutant plasmid compared to the wild-type lentivirus vector [wt(+dNTP)] are shown. The NLS mutant reduces transduction efficiency significantly compared to the wild-type [wt(−dNTP)] virus vector. The integrase mutant data are based on very few detectable cells. Improved efficiency of transduction in vivo with dNTP pretreatment (+dNTP) was apparent but did not reach statistical significance (ANOVA and Fisher's PLSD; $P < 0.05$). *, statistically significant.

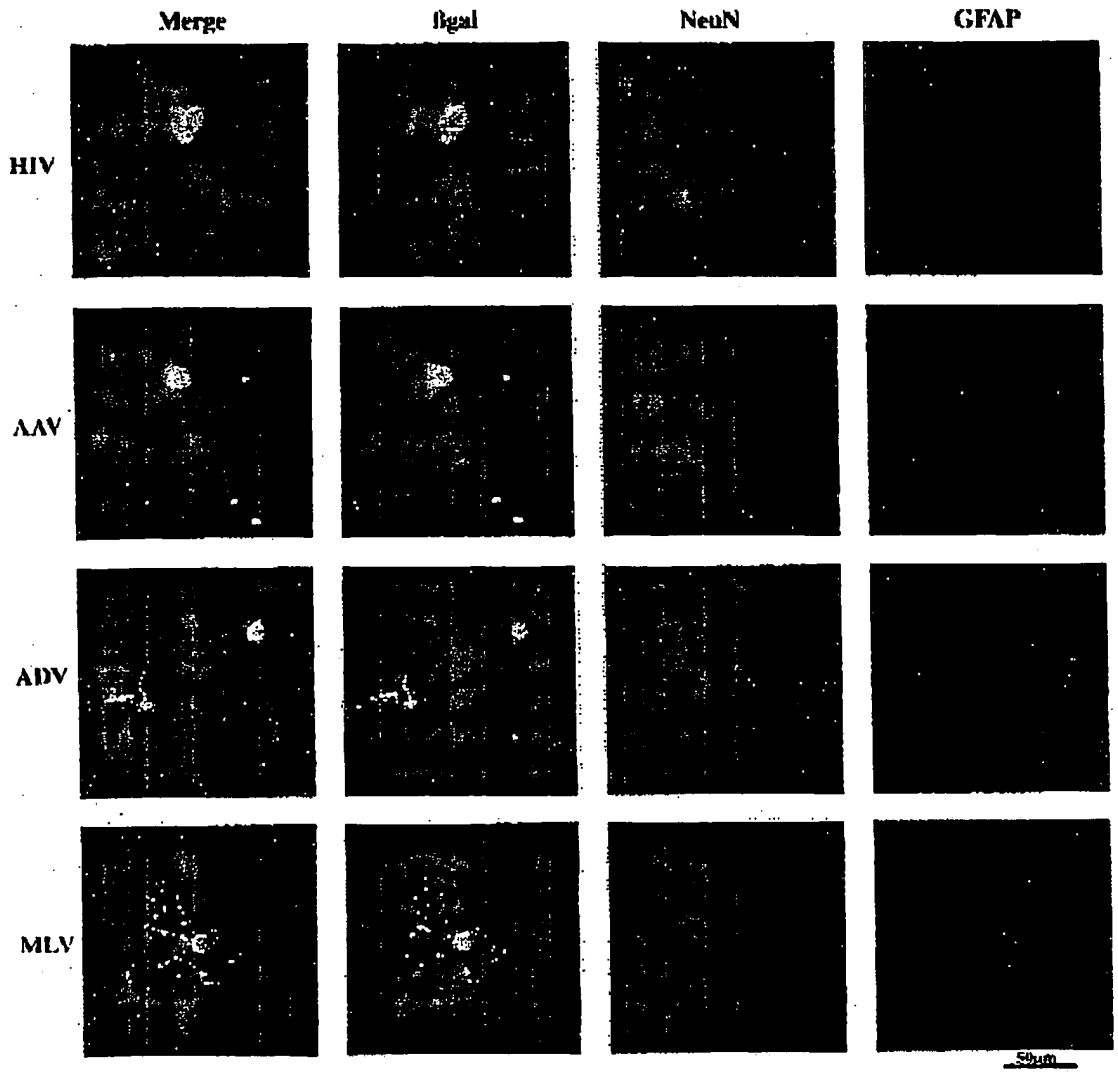

FIG. 4. In vivo transduction of adult rat central nervous system cells. Confocal microscope pictures of sections of β-Gal-expressing cells, transduced by the HIV, AAV, ADV, and MLV vectors, are shown. Immunofluorescence staining for the reporter gene product β-Gal (green), the neuron-specific marker NeuN (red), the glial fibrillary acidic protein GFAP (blue), and their overlap are shown for representative areas.

centration of dNTPs prior to infection of the cell (26, 39, 41, 56). The expression of transgenes was increased approximately 10-fold by pretreatment of the virions with dNTPs in vitro (71). Low dNTP concentrations in quiescent cells, such as terminally differentiated neurons in the central nervous system, are considered to be responsible for the inefficiency of retroviral infection in vivo (26, 41). Compartmentalization of the dNTPs in dividing cells is also believed to limit dNTP availability for viral DNA synthesis (38, 39, 56), although the promotion of reverse transcription by in vitro exposure to dNTP was shown to be more pronounced in nondividing than in dividing cells (48). However, the transduction efficiency of the lentivirus vector in our experiments did not show statistically significant improvement with dNTP pretreatment.

The main difference between lentivirus vectors and the conventional MLV-based vectors is their ability to transduce terminally differentiated cells. This reflects the recognition of the uncoated lentivirus nucleoprotein complex by the cell's nuclear import machinery and the active transport through the nucleopore (23–25, 40, 64) (Fig. 1). As expected the Vpr/MA NLS-defective lentivirus vector, in which Vpr and other karyophilic proteins involved in the nuclear translocation of the preintegration complex were inactivated, is less effective in the transfer of the transgene into target cells, and elimination of integrase activity via mutations in the catalytic domain of integrase eliminated transduction altogether. Recent results suggest that integrase not only mediates the integration of viral DNA into the host cell genome but is also involved in its transport to the

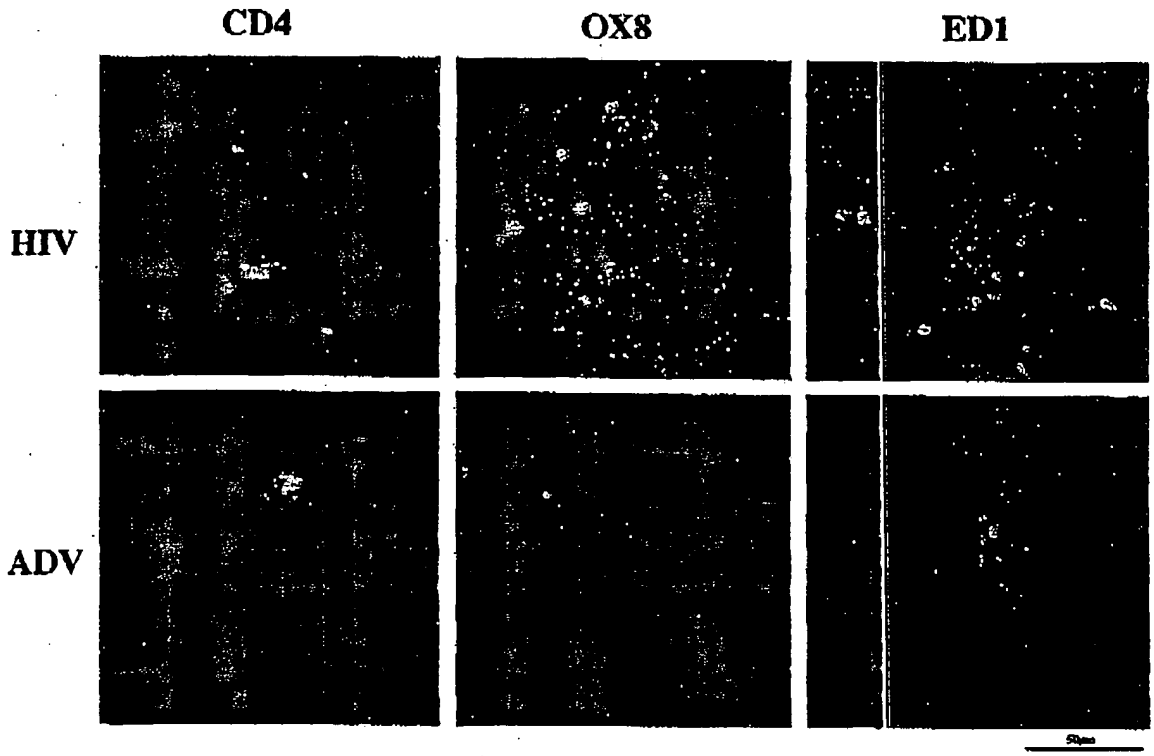

FIG. 5. Immune response in HIV vector- and ADV vector-injected brains. Immunohistochemical detection of lymphocytes and macrophages around the injection areas 6 weeks after striatal injection of HIV and ADV vectors is shown. CD4 lymphocytes (red) invade the injection site for the ADV vector, leaving very few cells expressing the β-Gal transgene (green). Cholinergic neurons are stained for Chat (blue). A large number of OX8-positive cells (red) in the ADV vector site compared to a single OX8 lymphocyte in the HIV vector site were found. Macrophages and microglial cells (red) in the injection tract are specific to HIV- and ADV-transduced cells (green).

nucleus. In the absence of integrase the HIV nucleoprotein complex lacks karyophilic potential (24, 25).

Transgene expression in a secondary area as a consequence of retrograde transport of the virus has been reported with replication-compromised (14) and replicating herpes simplex viruses (2, 18, 29, 67) and with replication-deficient adenovirus vectors (35, 67). The uptake of the virus at the striatal synapse, retrograde transport of the viral RNA, and subsequent stable integration into the genome of the secondary-area neuron result in stable expression of the transgene. The limited number of cells detected in secondary areas following the single injection of 2 μl of lentivirus vector into the primary areas, compared to the results obtained following infusion of rather large volumes of replication-competent self-amplifying vectors (adenovirus or herpes simplex virus), reflects the lesser degree of retrograde transport that occurs with a replication-deficient vector. In the present study we have shown that the nigrostriatal pathway is susceptible to lentivirus manipulation. A combination of transgene expression in primary injection areas and in secondary projection areas via retrograde transport may allow transduction of cells in target regions that are difficult to approach directly.

The central nervous system offers unique conditions for viral gene transfer, either direct transfer or indirect transfer via retrograde transport. The high differentiation and specification of brain areas frequently require only low levels of transgene expression to compensate for dysfunctions or to add and replace proteins, like dopamine in the Parkinsonian brain, at low levels. The blood-brain barrier not only protects the brain from extensive immune reactions but also allows regional transgene delivery. Many features make retroviral vectors a good choice for gene transfer in vivo. Most importantly the integration into

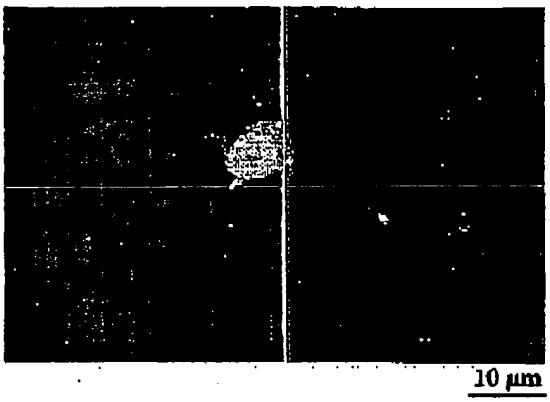

FIG. 6. Retrograde transport. Images obtained by confocal microscopy of horizontal sections through the brain stem 6 months after striatal injection with lentivirus vector expressing β-Gal (green) are shown. The reporter gene is detectable in the nigrostriatal pathway, with processes in the SN compacta and rare cells in the SN compacta expressing the transgene and tyrosine hydroxylase (red). Counter staining with Chat, marking cholinergic neurons (blue), is shown.

the target cell genome is efficient. The only other integrating viral vector derived from AAV is not efficient and does not appear to integrate into the host cell genome (34). Lentivirus vectors provide an efficient vehicle to stably integrate transgenes into dividing and nondividing cells without toxicity and immune response and with a stable high level of transgene expression over 6 months in vivo.

ACKNOWLEDGMENTS

We thank M. L. Gage, E. Makakis, T. D. Palmer, T. Bödefeld, and P. J. Horner for critical reviews of the manuscript.

U.B. was supported by the Deutsche Forschungsgemeinschaft, and L.N. was supported by the American-Italian Cancer Foundation. This work was supported by grants from the National Institutes of Health and the American Cancer Society (I.M.V.), U.S. Public Health Service grant AI 37510 (D.T.), National Institutes of Health grants AG 10435 and 08514 and the Hollfelder Foundation (F.H.G.), and the H. N. and Frances Berger Foundation (D.T., I.M.V., and F.H.G.).

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